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Purification and characterization of a novel thermal stable peroxidase from *Jatropha curcas* leaves

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ABSTRACT

A novel heme peroxidase from *Jatropha curcas*, an important source of bio-diesel, was purified to homogeneity using ammonium sulfate fractionation, desalting chromatography and ion exchange chromatography. Molecular mass of this purified enzyme was around 48 kDa as detected by SDS-PAGE. Gel filtration analysis revealed that the enzyme was a monomer under native conditions. The purified enzyme had broad substrate specificity with the ideal substrates of guaiacol and *o*-phenylenediamine. The optimum temperature, pH and K_m value of this peroxidase for guaiacol was 60 °C, 5.0 and 0.17 mM, respectively. In addition, NaCl (2.5 M) significantly enhanced the activity of this peroxidase. The purified enzyme was stable under high temperature (70% activity retained after 1 h incubation at 70 °C), extreme pH environment (93% or more activity retained after 2 h incubation under pH 3–12), high NaCl concentration (88% or more activity retained after 2 h incubation with 1–4 M NaCl) and organic solvents (95% or more activity retained after 54 h incubation with various organic solvents). Moreover, this peroxidase was resistant against 20 mM hydrogen peroxide, 8 M urea, 3 M guanidine hydrochloride and 20 mM EDTA. However, the peroxidase activity was significantly inhibited by sodium azide, dithiothreitol, CTAB, β-mercaptoethanol, DMSO, toluene and ferrous ion. The enzyme had long shelf life with 180 days at 4 °C and 14 days at room temperature. This new robust peroxidase may bring a better understanding for the high anti-adversity property of *J. curcas*. Meanwhile, the broad substrate specificity, wide stability against high temperature, extreme pH, organic solvent and hydrogen peroxide suggested that the enzyme could be a potential candidate peroxidase source for industrial and biomedical applications.

1. Introduction

Peroxidases (PODs, EC 1.11.1.7) are enzymes that oxidize various hydrogen donors in the presence of H_2O_2. They are widely existed in plants, animals and microorganisms. Plant PODs are involved in diverse physiological functions such as lignin biosynthesis [1], suberization [2], wound healing [3], fruit ripening [4], auxin metabolism and disease resistance [5].

Based on sequence homology and location, non-animal PODs have been classified into three classes: class I refers to intracellular POD (e.g., bacterial gene-duplicated catalase-peroxidase, yeast cytochrome C peroxidase and ascorbate peroxidase), class II includes secretory fungal PODs (e.g., lignin peroxidase and manganese peroxidase) and class III consists of the plant secretory PODs such as *Armoracia rusticana* peroxidase (HRP-C) and *Glycine max* peroxidase (SBP) [6]. The well known PODs are class III PODs, which share a heme prosthetic group, four conserved disulfide bridges, and two calcium binding sites [7].

PODs have been exploited for widespread applications such as enzymatic immunoassays, biosensors, clinical diagnostics and organic synthesis [5,8,9]. Meanwhile, they have also been widely used in detoxification of wastewater and industrial effluents because of their broad substrate specificity [10,11]. Over the years, *A. rusticana* (horseradish) peroxidases have been well studied and represent the only traditional commercial source of POD. Obviously, new an alternative source of PODs with better properties is needed. An ideal POD for industrial applications should be the one that is abundant, can be easily purified, shows broad substrate specificity and has better stability against high temperature and extreme pH conditions.

Jatropha curcas, well known as an important source of bio-diesel, is a drought-resistant plant that is widely distributed in Southeast Asia, Africa, India and South America [12]. *J. curcas* seeds contain above 40% viscous oil, which is suitable for bio-diesel, candles and high quality soap production [12]. After detoxification, the seed

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cakes obtained from oil extraction can be used as animal feed [13]. Because of the high anti-adversity properties, *J. curcas* can grow well on poor soils, making it easy to cultivate. Peroxidases of *J. curcas* have also been studied in recent years. It has been reported that *J. curcas* PODs are sensitive for salt stress [14] and metal stress such as mercury [15] and chromium [16]. In addition, *J. curcas* PODs are also involved in seed germination [17] and root elongation [18].

In this paper, we reported the purification and characterization of a new class III peroxidase from *J. curcas* leaves. Owing to its good thermal resistance, broad pH stability, high H_2O_2 tolerance and high organic solvent stability, this enzyme may become an ideal POD source for industrial and biomedical applications.

2. Materials and methods

2.1. Materials

J. curcas leaves were collected from several individual wild trees in Panzhihua city, Sichuan province, China. Guaiacol, ABTS, *o*-phenylenediamine, benzidine and HRP-C were purchased from Sigma–Aldrich. Blue dextran 2000, albumin, ovalbumin, chymotrypsinogen A, ribonuclease A, Sephadex G-25, Superdex-75 and HiTrap Q HP 5 ml prepacked column were obtained from GE Healthcare. Other reagents and chemicals were of analytical grade.

2.2. Purification of peroxidase

2.2.1. Crude extracts preparation and ammonium sulfate fractionation

J. curcas leaves (400 g) were cut into pieces and homogenized in 2.4 l buffer A (50 mM Tris–HCl, pH 8.0). After 12 h stirring at 4 °C, the homogenate was filtered through carbasus and centrifuged at 10,000 × *g* for 30 min. Supernatant was collected and brought to 50% saturation of ammonium sulfate followed by stirring at 4 °C for 5 h. Then, the solution was centrifuged at 10,000 × *g* for 30 min and the supernatant was precipitated by increasing the ammonium sulfate saturation up to 85%. After stirring at 4 °C for 12 h, the solution was centrifuged at 10,000 × *g* for 30 min and pellet was dissolved in 80 ml buffer A for further purification.

2.2.2. Chromatography procedure

To further purify the target protein, desalting chromatography and anion-exchange chromatography were processed on AKTA Purifier UPC 10 (GE Healthcare) in a cold room. During the purification, proteins were monitored at 280 nm, salt concentration was monitored by conductivity sensor and the flow-rates were kept at 150 cm/h.

An XK 26/20 column (GE Healthcare) packed with 54 ml of Sephadex G-25 resin was used for desalting. Dissolved precipitates (see above) were loaded onto the desalting column (pre-equilibrated with buffer A) and eluted by buffer A using isocratic elution modern. Fractions showing POD activity were collected, pooled and further injected into a HiTrap Q HP 5 ml prepacked column (pre-equilibrated with buffer A). After washing with buffer A, the bound proteins were eluted by three-step gradients (0.1 M, 0.6 M, and 1 M NaCl in buffer A, respectively). Fractions showing POD activity were collected and used for further experiments.

2.3. SDS-PAGE and Native-PAGE electrophoresis

SDS-PAGE was performed in 12% gel. Purified enzymes (20 μg) were loaded. Gels were stained with Coomassie Blue R-250.

Native-PAGE was performed under similar condition without SDS. Electrophoresis was carried out at 4 °C in 10% gels. Purified enzymes (10 μg) were loaded. Gels were then stained with Coomassie Blue R-250 or POD activity detecting solution (20 mM benzidine, 0.03% H_2O_2, 280 mM acetic acid, 60 mM NH_4Cl and 10 mM EDTA).

2.4. Protein determination

Protein concentration was measured according to the procedure described by Bradford [19]. Bovine serum albumin (BSA) was used as standard protein.

2.5. Enzyme characterization

2.5.1. Molecular mass determination

The molecular mass of the native enzyme was determined by gel filtration on a Superdex-75 column (1.6 cm × 65 cm). Buffer was the same as that used in desalting. Albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) were used as molecular weight (MW) standards. Void volume (V_o) of the column was measured by blue dextran. V_e represented elution volume and V_e/V_o was defined as R_f value. The $\log MW - R_f$ graph obtained from above standards was used to determine the native molecular mass of purified enzyme.

2.5.2. UV–Vis spectra and atomic absorption analysis

Purified enzyme was dissolved in water (200 μg/ml) and scanned between 260 nm and 700 nm on a spectrophotometer (UV-8100; Lab-Tech). Ferrum and calcium concentrations were determined by atomic absorption spectrometry (SpectrAA 220Z; Varian). Standard solutions of these two metal ions were used for quantitation.

2.5.3. Substrate specificity, optimum pH and peroxidase activity assay

Substrate specificity was studied by spectrophotometric assay using H_2O_2 in combination with various substrates. The following wavelengths and molar extinction coefficients were used for each substrate: guaiacol, 470 nm, $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$; *o*-phenylenediamine, 445 nm, $\varepsilon = 11.1\ mM^{-1}\ cm^{-1}$; *o*-dianisidine, 460 nm, $\varepsilon = 30\ mM^{-1}\ cm^{-1}$; ABTS, 414 nm, $\varepsilon = 36\ mM^{-1}\ cm^{-1}$; pyrogallol, 420 nm, $\varepsilon = 2.4\ mM^{-1}\ cm^{-1}$; ferulic acid, 318 nm, $\varepsilon = 16.6\ mM^{-1}\ cm^{-1}$. To study the kinetic parameters, different concentrations of each substrate were added in 4 ml of reaction solution containing 50 mM appropriate buffer, 100 μl of appropriately diluted enzyme and 15 mM H_2O_2. Oxidation rates of each hydrogen donors were measured in terms of the increase or decrease in absorption at specific wavelength for 1.5 min at 25 °C. The Michaelis constant (K_m) and maximum catalytic rate (V_{max}) were calculated from Lineweaver–Burk plotting. Specific activity for each substrate was defined as the amount of substrate that was oxidized per min by 1 mg purified enzyme.

Optimal pH for each substrate was determined by measuring the oxidation rates at 25 °C under the following buffers from pH 2.5 to 9.0: 50 mM citrate buffer for pH 2.5–5.5; 50 mM phosphate buffer for pH 6.0–7.5; 50 mM Tris–HCl buffer for pH 8.0–8.5; 50 mM glycine–NaOH buffer for pH 9.0. Three independent experiments were done for each measurement.

After the substrate preference study, guaiacol was chosen as the substrate for the peroxidase activity assay. The reaction was initiated by adding 50 ng enzymes into 4.0 ml reaction mixture contained 15 mM H_2O_2, 10 mM guaiacol, and 50 mM citrate buffer (pH 5.0). Absorbance changes at 470 nm were monitored for 1.5 min at 25 °C. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of guaiacol per min at 25 °C.

Table 1
Summary of each purification step of peroxidase from *Jatropha curcas* leaves.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	2880	330,000	115	1	100
85% ammonium sulfate precipitation	120	306,000	2550	22.2	92.6
Sephadex G-25 desalting	98.4	271,000	2770	24.1	82.1
Q-Sepharose HP anion exchange	5.0	124,000	24,800	216	37.6

2.5.4. Determination of pH stability

pH stability profile was investigated by incubating the purified enzyme with different pH buffers for 2 h at room temperature. Then the residual activity was measured under standard conditions as described in Section 2.5.3. Assays were repeated three times.

2.5.5. Determination of optimum temperature, thermal stability and storage stability

The optimal temperature was determined by measuring enzyme activity under different temperature from 20 to 90 °C. In order to determine the thermal stability of the purified *J. curcas* POD or HRP-C, the enzyme solution was incubated at various temperatures (40–80 °C) for different times (0–60 min). After the incubation, the enzyme was cooled in ice bath for 30 min following residual activity assay as described above. For storage stability determination, purified enzyme (0.5 mg/ml) was first stored at 25 °C, 4 °C and −80 °C for 180 days in buffer B (50 mM Tris–HCl, 100 mM NaCl, pH 8.0) and then the residual activity was measured as described above. All above experiments were repeated three times.

2.5.6. Determination of optimum ionic strength and salt tolerance

The optimum ionic strength was studied by adding different concentrations of NaCl into the standard reaction mixture. The activity assays were performed as described in Section 2.5.3. To investigate the salt tolerance, purified enzyme (50 ng) was first incubated with various concentrations of NaCl for 2 h at room temperature and then the residual activity was assayed under NaCl free conditions as described in Section 2.5.3. All above experiments were repeated three times.

2.5.7. Effect of various compounds

Purified enzyme (50 ng) was incubated for 2 h at 25 °C with various compounds (H_2O_2, guanidine hydrochloride, urea, EDTA, SDS, Triton X-100, sodium azide, DTT, CTAB, β-mercaptoethanol, citric acid and oxalic acid). After incubation, the residual activity was assayed as described above. All experiments were repeated three times.

2.5.8. Effect of metals ions

Purified enzyme (50 ng) was pre-incubated for 2 h at 25 °C with various metal ions (Pb^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Ba^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Li^{+}, Fe^{2+}, Cd^{2+} and Mg^{2+}) following residual activity measurement. Assays were repeated three times.

2.5.9. Effect of organic solvents

Purified enzyme (50 ng) was incubated in various organic solvents (25%, v/v) for 2–54 h at 25 °C with constant shaking at 150 rpm. After incubation, residual activity was measured as described above. Assays were repeated three times.

3. Results and discussion

3.1. Purification of the enzyme

Although several peroxidase isoenzymes were observed in crude extract of *J. curcas* leaves (data not shown), we focused on the most abundant isoenzyme in this study. One POD isoenzyme, named JcGP1, was successfully purified through three steps: ammonium sulfate precipitation, Sephadex G-25 desalting and Q-Sepharose HP anion exchange chromatography. The results for each purification step are summarized in Table 1.

Crude extracts of *J. curcas* leaves were precipitated by ammonium sulfate. JcGP1 was found to be precipitated in the range of 50–85% saturation while bulk impurities were removed by 0–50% precipitation. After this step, 22 purification fold and 92.6% yield were obtained (Table 1). High recovery of this step suggested that treatment of crude extract with ammonium sulfate up to 85% saturation did not cause obvious loss of POD activity. The pellets obtained from 50 to 85% precipitation were redissolved and loaded onto a Sephadex G-25 column for desalting and buffer exchange. JcGP1 was found in the first A_{280} peak and low molecular weight impurities were removed simultaneously. The desalted sample was pooled and injected in a Q-Sepharose HP column for polishing. After washing away unbound protein, JcGP1 was eluted by 0.1 M NaCl while other impurities were observed in the 0.6 M NaCl fractions. After this final purification step, 216 fold was achieved with a yield of 37.6%. The specific activity of the purified POD was 24,800 U/mg (Table 1). The purified JcGP1 showed one band under denaturing or non-denaturing conditions, indicating the high purity and homogeneity (Fig. 1a and b).

3.2. Enzyme characterization

3.2.1. Determination of molecular weight

The molecular weight (MW) of purified JcGP1 was analyzed by gel filtration and SDS-PAGE. After Superdex-75 chromatography, a sharp peak around 48 kDa was detected (Fig. 1c). Single band with corresponding MW was also observed in SDS-PAGE analysis (Fig. 1a). Collectively, these results indicated that native JcGP1 is a monomer protein with the MW of ~48 kDa, which was similar with the MW reported for PODs from *Brassica rapa* (49 kDa) [20], *Viscum angulatum* (46.4 kDa) [21] and *Brassica oleracea* var. *italica* (48 kDa) [22]. MW of most plant PODs varies from 30 to 60 kDa, which is attributed to the difference of amino acid sequence as well as different level of glycosylation [6]. Notably, a 6 kDa peptide with POD activity is isolated from *Raphanus sativus* [23]. The majority of PODs reported to date are monomers. However, homotetramer PODs are also observed in *Vanilla planifolia* [24] and *Elaeis guineensis* [25].

3.2.2. UV–Vis spectra and atomic absorption analysis

A Soret band at 402 nm and two small peaks at 498 and 637 nm were observed in the UV–Vis spectrum (Fig. 2), suggesting JcGP1 was a heme protein. Similar results are also reported for other class III PODs such as *Ipomoea carnea* POD (401, 498 and 637 nm) [26] and HRP-C (402.5, 498 and 633 nm) [27]. RZ value (A_{402}/A_{280}) calculated from UV–Vis spectrum was 3.2, confirming a high purity of JcGP1. Atomic absorption analysis showed that the contents of ferrum and calcium atoms in JcGP1 were approximately 1.1 μg per mg protein and 1.6 μg per mg protein, respectively. The above results suggested that one JcGP1 protein contained one heme group and two calcium binding sites, corresponding to the typical structural feature of class III peroxidase [6].

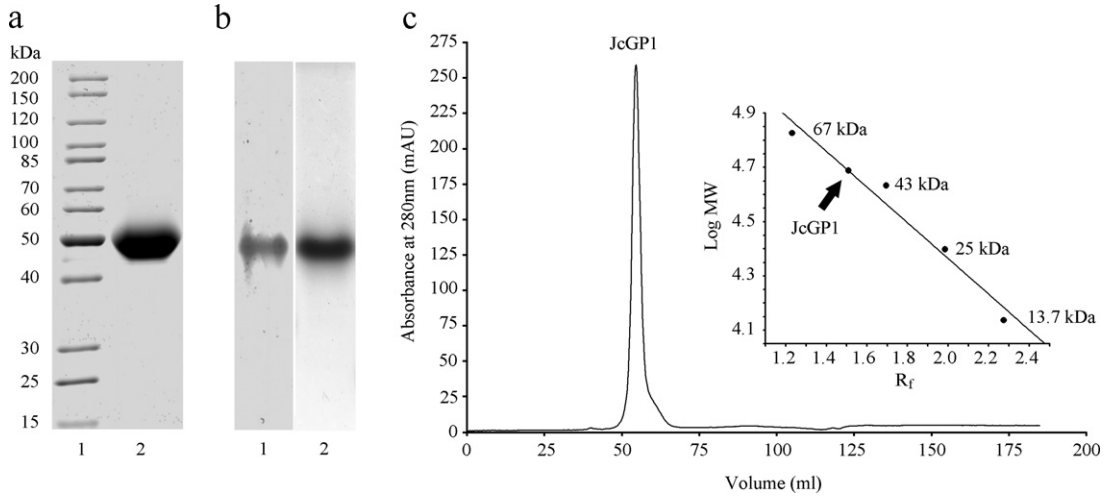

Fig. 1. (a) SDS-PAGE analysis of purified JcGP1 with Coomassie Blue staining. Lane 1: protein molecular weight marker; Lane 2: purified JcGP1. (b) Native-PAGE analysis of purified JcGP1. Lane 1: POD activity staining; Lane 2: Coomassie Blue staining. (c) Molecular weight determination of JcGP1 using gel filtration. Markers were used to obtain the log MW – R_f standard graph. Arrowhead indicated R_f position of JcGP1.

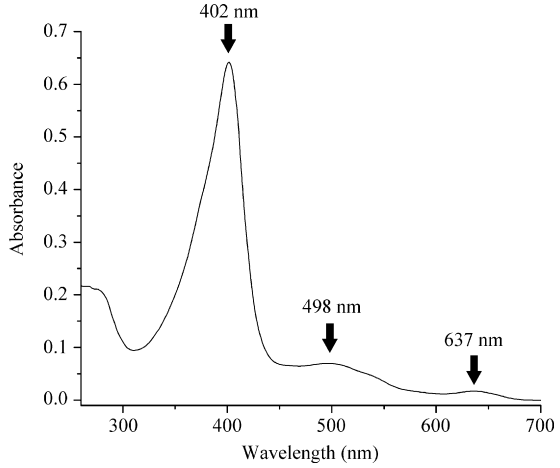

Fig. 2. UV–Vis spectrum of purified JcGP1. JcGP1 was dissolved in water and scanned from 260 to 700 nm on a UV–Vis spectrophotometer. The RZ value (A_{402}/A_{280}) was calculated as 3.2 from the spectrum. Peaks at 402, 498 and 637 nm were marked with arrowhead.

3.2.3. Determination of optimum pH and substrate specificity

Like many other plant PODs, JcGP1 was able to oxidize many well known POD substrates such as guaiacol, *o*-phenylenediamine, *o*-dianisidine, ABTS, pyrogallol and ferulic acid. The optimum pH for most of these substrates, excepted for pyrogallol, ranged from 3.5 to 5.0 (Table 2), revealing a preference for acidic environment. Activity for each substrate was decreased sharply when environment pH was out of the optimal range (data not shown). Many other reported plant PODs also have their highest activity in acidic condition. For example, *Vigna mungo* POD and *Momordica charantia* POD share a same optimum pH of 5.5 [28,29]. *Nicotiana tabacum* POD and *V. planifolia* POD have their highest activity at 4.5 and 3.8, respectively [24,30].

Table 2
Substrate specificity of JcGP1.

Substrate	Optimum pH	λ (nm)	K_m (mM)	Specific activity (μmol min^{-1} mg^{-1})
Guaiacol	5.0	470	0.17	24,800
o-Phenylenediamine	5.0	445	0.67	24,600
o-Dianisidine	4.5	460	2.45	19,100
ABTS	3.5	414	0.19	16,300
Pyrogallol	7.0	420	7.36	6070
Ferulic acid	4.0	318	8.48	4800

For substrate specificity study, K_m and specific activity of each substrate were measured at 25 °C under optimum pH. Results are summarized in Table 2. Among these six substrates, guaiacol, ABTS, and *o*-phenylenediamine showed high affinity toward JcGP1 with the K_m value of 0.17 mM, 0.19 mM and 0.67 mM, respectively. K_m values of other substrates were 15–50 folds higher than that of guaiacol, indicating relatively low affinity. As shown in Table 2, JcGP1 had high specific activity toward many substrates, suggesting broad substrate specificity. The specific activity of these substrates decreased in the order of guaiacol ≈ *o*-phenylenediamine > *o*-dianisidine > ABTS > pyrogallol > ferulic acid. Thus the best substrates for JcGP1 were guaiacol and *o*-phenylenediamine. The broad substrate specificity revealed that JcGP1 had potentials for industrial application such as wastewater treatment [10] and industrial effluents detoxification [11]. Feruloyl residues, which provide rigidity to the cell wall, can form oligomer with each other via oxidative coupling catalyzed by peroxidase. This oligomerization strengthens the cell wall, protecting it from digestion, and bringing resistance to pathogen invasion [31]. Therefore, the ability of utilizing ferulic acid as substrate indicated that JcGP1 may be involved in these mentioned physiological functions. In addition, the preference of this peroxidase for aromatic electron donors (such as guaiacol, *o*-phenylenediamine, *o*-dianisidine and ferulic acid) suggested that JcGP1 could belong to guaiacol peroxidase (GPOX), which has a role in lignin biosynthesis and hyperosmotic stress response [32].

3.2.4. Determination of pH stability

JcGP1 was stable in a broad pH range. More than 93% of maximum activity was kept after 2 h incubation with different pH buffers ranging from 3 to 12 (Fig. 3). It has been reported that many class III PODs lose their activities under extreme pH conditions and the loss of activity might be due to the detachment of the heme group [33]. The high resistance against low pH suggested that JcGP1 may hold its heme group more tightly than many other plant PODs. *G. max* POD and *Roystonea regia* POD have also been reported to exhibit good stability under low pH conditions [34,35]. The low optimum pH and high resistance against acidic conditions suggested that JcGP1 could be a suitable candidate POD source for dye degradation (e.g., romazol blue) [36] and conducting

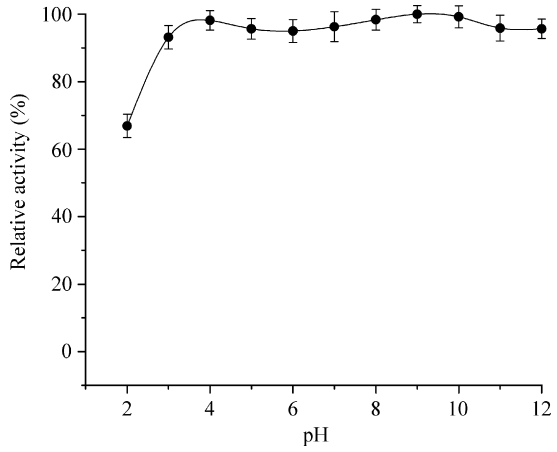

Fig. 3. pH stability of JcGP1. Purified JcGP1 was first incubated for 2 h under various pH values (pH 2–12) and then the residual activity was measured using guaiacol as the substrate. The highest activity was set as 100%.

polyaniline synthesis [37], which require PODs to work under low pH conditions.

3.2.5. Temperature activity profile

As shown in Fig. 4a, the maximum activity for JcGP1 was found to be at 60 °C. Moreover, it is remarkable that JcGP1 exhibited more than 90% of maximum activity under high temperature ranging from 55 °C to 75 °C. This high temperature preference suggested that JcGP1 may be considered as a heat-induced POD in *J. curcas*. Similar high optimum temperature is also observed in some other plant PODs such as *B. oleraceae* L. POD (57 °C) [38] and *Solanum melongena* POD (84 °C) [39].

Although the optimum temperature of JcGP1 (60 °C) was too high for most applications of peroxidase, the reasonable high specific activity for several common peroxidase substrate under 25 °C (Table 2) was acceptable for many potential industrial and biomedical applications. As we know, *J. curcas* is widely distributed in semitropics and dry-hot river valley. Thus, the hot preference of JcGP1 could maintain its physiological functions via retaining peroxidase activity under high temperature, which could be one of the reasons for the high adaptability of *J. curcas* for hot climate.

3.2.6. Determination of thermal stability and storage stability

For this study, purified enzyme was incubated for 0–60 min under various temperatures. As shown in Fig. 4b, JcGP1 was stable up to 70 °C. After 10 min incubation, almost 100% activity was kept at 40–60 °C, and 83% activity remained at 70 °C. After 60 min incubation, full activity was still retained at 40–50 °C, with 85% activity at 60 °C and 70% at 70 °C. A sharp loss of activity was observed after 10 min incubation at 80 °C and no activity was detected after 30 min incubation.

The thermal stability of JcGP1 was greater than many other reported plant PODs such as *V. planifolia* [24] and *Fragaria* × *ananassa* [40]. A comparison of thermal stability between JcGP1 and HRP-C, a traditional commercial source of POD, also indicated that JcGP1 was more stable than HRP-C at 50 °C and 70 °C (Fig. 4c and d). Owing to the high optimum temperature and high thermal stability, JcGP1 may be suitable for industrial applications, which require PODs to work under high temperature. Similar high

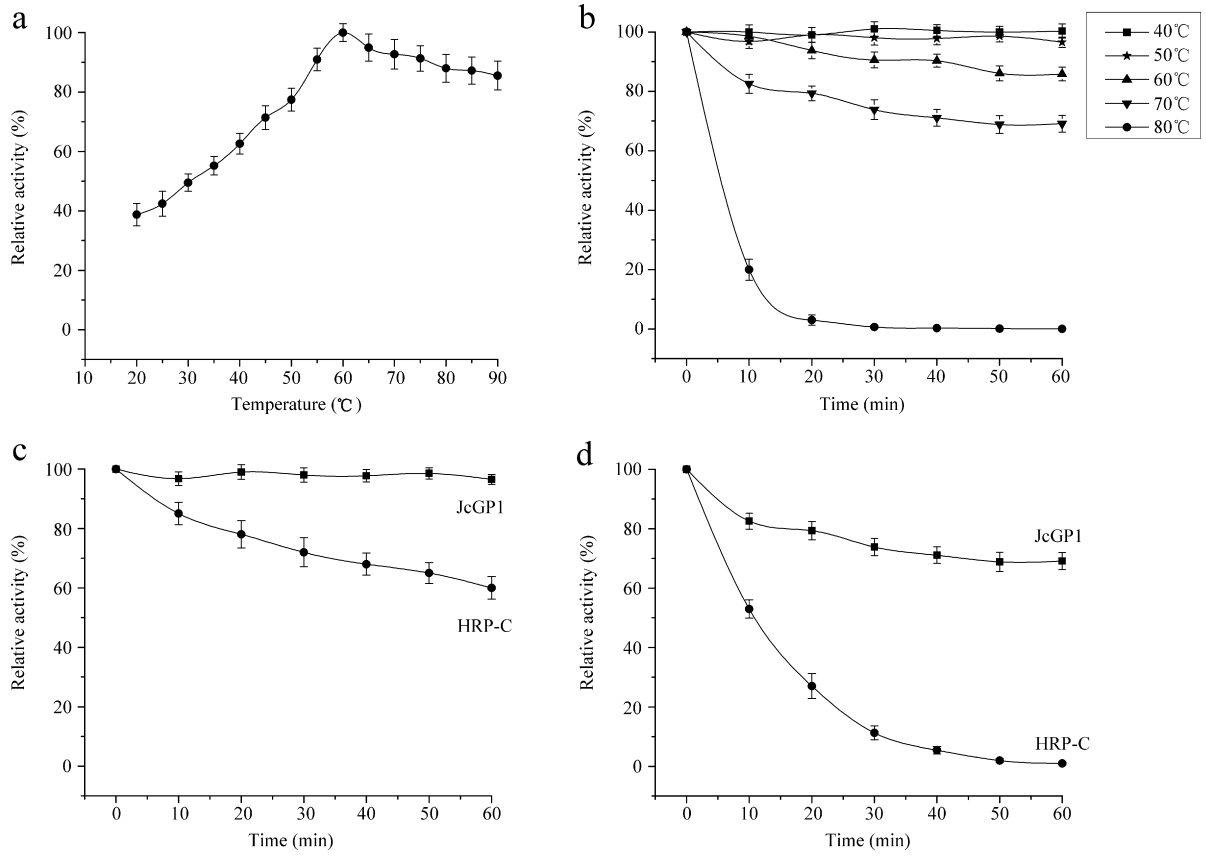

Fig. 4. (a) Effect of temperature on the POD activity of JcGP1. POD activity was measured under various temperatures (20–90 °C) using guaiacol as the substrate. The highest activity was set as 100%. (b) Thermal stability of JcGP1. Purified JcGP1 was first incubated for 0–60 min under 40 °C (■), 50 °C (★), 60 °C (▲), 70 °C (▼) and 80 °C (●), then the residual activity was measured using guaiacol as the substrate. Activity of the enzyme without heating was set as 100%. Comparison of thermal stability between JcGP1 (■) and HRP-C (●) under 50 °C (c) and 70 °C (d).

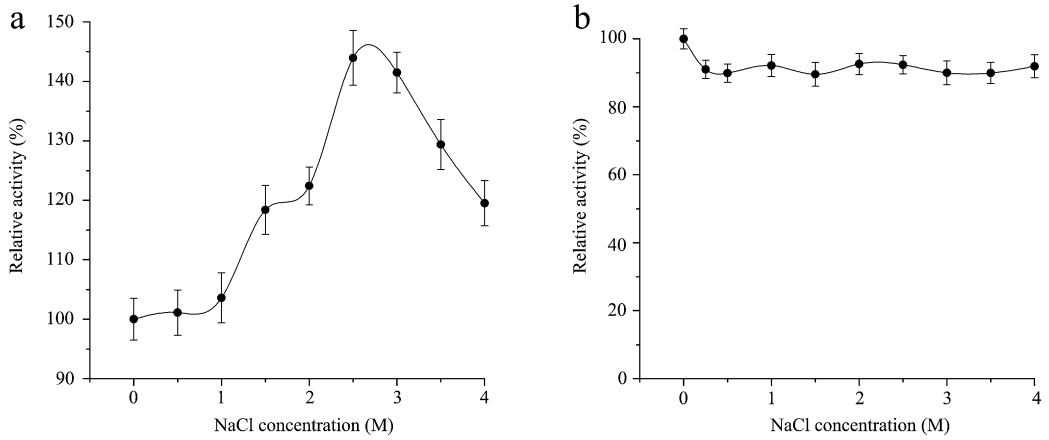

Fig. 5. (a) Optimum ionic strength of JcGP1. POD activity was measured under conditions with 1–4 M NaCl using guaiacol as the substrate. Activity of the enzyme under conditions without NaCl was set as 100%. (b) Salt tolerance of JcGP1. Purified JcGP1 was first incubated for 2 h with various concentrations of NaCl and then the residual activity was measured under NaCl free conditions using guaiacol as the substrate. Activity of the enzyme incubated without NaCl was set as 100%.

thermal stability is also observed in some other plant PODs such as *V. angulatum* POD [21] and *I. carnea* POD [26].

In addition, the shelf life of the enzyme was studied. JcGP1 solution was stable under −80 °C and 4 °C with almost full activity remained after 180 days storage. Further more, it was remarkable that JcGP1 solution can even be left at room temperature for 14 days with 93% of activity remained. The unusual long shelf life and the high thermal stability of JcGP1 may broaden its applications in biomedicine, biosensor and many other areas mentioned before.

3.2.7. Optimum ionic strength and salt tolerance determination

Optimum ionic strength was analyzed by adding NaCl into the reaction mixture. As shown in Fig. 5a, high concentration of NaCl significantly enhanced the POD activity of JcGP1. The optimum concentration of NaCl was 2.5 M with the POD activity enhancement of 44%. The mechanism of how salt can enhance POD activity of JcGP1 was unknown. However, similar results have been reported in both *R. sativus* L. POD [41] and *Leucaena leucocephala* POD [42], with the optimum ionic strength of 1 M Na_2SO_4 and 3 M NaCl, respectively. In addition, the activities of HRP-C and SBP in some anhydrous solvents are also increased by salt activation [43]. To investigate the salt tolerance, JcGP1 was first incubated with various concentrations of NaCl for 2 h and then the residual activity was measured under NaCl free conditions. As shown in Fig. 5b, more than 88% activity was retained after 2 h incubation with up to 4 M NaCl, suggesting high salt tolerance of JcGP1.

All the results above indicated that JcGP1 exhibited significantly higher activities under the reaction conditions containing NaCl than conditions without NaCl. Meanwhile, the enzyme was stable after incubation with high concentration of NaCl for 2 h. Collectively, the peroxidase stability of JcGP1 against hyperosmotic condition, extreme pH, and high temperature might be responsible for the high anti-adversity of *J. curcas*.

3.2.8. Effect of various compounds on peroxidase activity

Table 3 shows the details of inhibitions of JcGP1 by different compounds. It was found that the enzyme was resistant against 1–20 mM H_2O_2, 0.75–3 M guanidine hydrochloride (GdnHCl), 2–8 M urea and 1–20 mM EDTA. Low concentration (0.5%) of SDS and Triton X-100 slightly increased the enzyme activity while high concentration (4%) showed 10.1% and 19.8% inhibition, respectively. As many other class III peroxidases, the activity of JcGP1 was significantly inhibited by sodium azide (NaN_3) and dithiothreitol (DTT), and the inhibition was almost 100%. The inhibitory effect of CTAB and β-mercaptoethanol (β-ME) was concentration dependent and the highest inhibition by 20 mM CTAB and β-ME was 90.2% and 83.6%, respectively. Unlike *V. angulatum* POD [21] and *V. mungo* POD [28], 5–20 mM citric acid and oxalic acid had no significant effect on JcGP1. The inhibitory effect of DTT and β-ME suggested that disulfide bonds within the structure of JcGP1 was responsible for its peroxidase activity, which was in correspondence with the structure characterization of class III peroxidase [7]. In addition, high tolerance against 8 M urea is also observed in *M. charantia* POD [29].

Many peroxidases, such as HRP and SBP, were intolerant against high concentration of H_2O_2. The relative activity for HRP and SBP under 10 mM H_2O_2 was below 20% and 50%, respectively [44]. This inhibition by high concentration of H_2O_2 is one of the drawbacks that limit HRP and SBP applications [8]. The significantly higher tolerance against H_2O_2 made JcGP1 more suitable for industrial or

Table 3
Percentage of inhibitions (%) of JcGP1 activity by various compounds. Each compound was tested under three different concentrations. Results were shown as mean ± standard deviation of three replicas.

Compounds	Conc. 1	Inhibition (%)	Conc. 2	Inhibition (%)	Conc. 3	Inhibition (%)
H_2O_2	1 mM	6.5 ± 2.4	10 mM	7.2 ± 3.4	20 mM	7.1 ± 3.2
GdnHCl	0.75 M	4.6 ± 2.9	1.5 M	3.4 ± 2.7	3 M	3.2 ± 3.5
Urea	2 M	0.6 ± 2.1	4 M	1.2 ± 1.9	8 M	1.3 ± 2.1
EDTA	1 mM	−1.2 ± 2.8	10 mM	−0.8 ± 2.5	20 mM	−1.1 ± 2.7
SDS	0.5% (m/v)	−5.3 ± 2.3	2% (m/v)	3.4 ± 1.9	4% (m/v)	10.1 ± 2.2
Triton X-100	0.5% (v/v)	−11.3 ± 3.1	2% (v/v)	5.6 ± 2.9	4% (v/v)	19.8 ± 2.7
NaN_3	1 mM	36.9 ± 3.5	5 mM	95.7 ± 1.2	10 mM	98.8 ± 0.8
DTT	1 mM	95.1 ± 0.7	5 mM	98.7 ± 1.0	10 mM	99.0 ± 0.6
CTAB	5 mM	67.5 ± 2.1	10 mM	79.4 ± 2.8	20 mM	90.2 ± 1.2
β-ME	5 mM	51.3 ± 3.2	10 mM	62.2 ± 2.9	20 mM	83.6 ± 1.8
Citric acid	5 mM	0.3 ± 1.5	10 mM	0.9 ± 1.7	20 mM	−1.1 ± 1.4
Oxalic acid	5 mM	1.2 ± 2.0	10 mM	0.6 ± 1.8	20 mM	1.4 ± 2.0

Table 4
Effect of metal ions on JcGP1. Purified JcGP1 was first incubated with 1, 5 and 10 mM various metal ions for 2 h and then the residual activity was measured. Activity of the enzyme without metal ions incubation (control) was set as 100%. Results were shown as mean ± standard deviation of three replicas.

Compound	Residual activity (%)		
	1 mM	5 mM	10 mM
Control	100	100	100
$(CH_3COO)_2Pb$	105.6 ± 4.3	114.6 ± 5.2	109.9 ± 4.8
$Al(NO_3)_3$	106.8 ± 3.9	109.2 ± 3.5	97.3 ± 5.1
$ZnSO_4$	106.1 ± 5.4	118.4 ± 4.7	107.8 ± 5.2
$CuCl_2$	113.6 ± 3.6	110.9 ± 4.4	105.8 ± 3.8
$NiSO_4$	99.6 ± 3.0	104.7 ± 3.2	98.2 ± 4.3
$BaCl_2$	130.8 ± 6.5	124.0 ± 5.8	118.3 ± 4.9
$CoCl_2$	95.7 ± 4.0	98.6 ± 3.3	104.3 ± 3.7
$CaCl_2$	100.2 ± 3.1	96.4 ± 2.9	92.1 ± 3.5
$MnSO_4$	100.5 ± 5.3	89.2 ± 4.8	89.6 ± 5.2
LiCl	96.8 ± 3.4	96.8 ± 3.1	95.7 ± 2.8
$FeCl_2$	77.8 ± 5.9	67.7 ± 6.4	59.7 ± 6.6
$CdCl_2$	105.0 ± 3.5	102.2 ± 5.1	94.6 ± 4.4
$MgCl_2$	101.4 ± 3.8	96.4 ± 2.9	88.9 ± 4.2

biomedical applications, which require peroxidase to work under high concentration of H_2O_2.

3.2.9. Effect of metals ions on peroxidase activity

JcGP1 was incubated with various metal ions and the residual activity is shown in Table 4. POD activity was slightly increased by 5 mM Pb^{2+}, 5 mM Zn^{2+} and 1 mM Cu^{2+}. Mn^{2+} (10 mM) and Mg^{2+} (10 mM) showed low inhibition. Al^{3+}, Ni^{2+}, Co^{2+}, Li^{+} and Cd^{2+} had no significant effect on POD activity. About 30% activity increase was observed in 1 mM Ba^{2+} while 40% inhibition was caused by 10 mM Fe^{2+}. Strong inhibition by Fe^{2+} was also observed in *V. planifolia* POD [24].

3.2.10. Effect of organic solvents on peroxidase activity

JcGP1 was stable against many organic solvents. As shown in Table 5, more than 95% of full activity was retained after 54 h incubation with methanol, acetone, isopropanol, *n*-hexane, isooctane, ethanol, acetonitrile, acetoacetate, glycerol, 1-butanol and *n*-heptane. Furthermore, incubation with ethanol, acetonitrile, acetoacetate, glycerol, 1-butanol and *n*-heptane significantly activated POD activity. This enhancement may be explained that organic solvent molecules could interact with hydrophobic amino acid residues of JcGP1, resulting in a more suitable conformation for catalysis. Moreover, JcGP1 POD activity was rapidly decreased after 2 h of incubation with DMSO and toluene. Complete inhibitions were observed after 6 h of incubation.

Table 5
Effect of organic solvent on JcGP1. Purified JcGP1 was first incubated for 2–54 h with various organic solvents (25%, v/v) and then the residual activity was measured. Activity of the enzyme without organic solvent incubation (control) was set as 100%. Results were shown as mean ± standard deviation of three replicas.

Organic solvents	Residual activity (%)		
	2 h	6 h	54 h
Control	100	100	100
Methanol	102.5 ± 3.5	99.2 ± 3.3	96.5 ± 3.8
Acetone	98.3 ± 2.9	97.7 ± 2.5	97.4 ± 3.2
Isopropanol	103.3 ± 4.3	97.3 ± 3.1	100.6 ± 3.7
n-Hexane	98.5 ± 4.4	99.3 ± 4.1	99.1 ± 4.5
Isooctane	99.6 ± 2.4	95.3 ± 3.8	94.2 ± 2.8
Ethanol	107.3 ± 1.7	108.2 ± 2.2	108.7 ± 1.3
Acetonitrile	108.9 ± 1.6	107.6 ± 1.8	105.3 ± 2.0
Acetoacetate	130.3 ± 2.3	114.5 ± 2.5	116.2 ± 2.9
Glycerol	116.5 ± 3.0	114.5 ± 3.7	112.4 ± 2.8
1-Butanol	140.3 ± 4.4	130.5 ± 3.0	126.3 ± 3.6
n-Heptane	117.8 ± 2.8	116.0 ± 2.1	117.0 ± 2.5
DMSO	25.8 ± 1.2	0.8 ± 0.2	0.6 ± 0.1
Toluene	71.5 ± 3.2	2.8 ± 0.7	1.3 ± 0.4

4. Conclusion

A novel peroxidase from *J. curcas* leaves was purified to homogeneity by three steps. The purified enzyme showed broad substrate specificity, high thermal stability, wide pH resistance, high salt and H_2O_2 tolerance, long shelf life and good stability against many organic solvents, which are important properties for industrial and biomedical applications. Further studies may focus on the mechanism of thermal stability and salt activation of JcGP1 as well as chemical modification of the enzyme for stability improvement. Moreover, because *J. curcas* leaves are abundant, can be easily collected and are not raw materials for bio-diesel production, the JcGP1 purification procedure could be further optimized and integrated into the *J. curcas* bio-diesel industrial chain. JcGP1 might be developed as a byproduct of *J. curcas* bio-diesel.

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